Transduction Efficiencies of Novel AAV Vectors in Mouse Airway Epithelium *In Vivo* and Human Ciliated Airway Epithelium *In Vitro*

Maria P Limberis[1], Luk H Vandenberghe[1], Liqun Zhang[2,3], Raymond J Pickles[2,3] and James M Wilson[1]

[1]*Gene Therapy Program, Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, USA;* [2]*Cystic Fibrosis/Pulmonary Research and Treatment Center, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA;* [3]*Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA*

We have characterized the ability of adeno-associated virus (AAV) serotypes 1–9 in addition to nineteen novel vectors isolated from various tissues, to transduce mouse and human ciliated airway epithelium (HAE). Vectors expressing α-1-antitrypsin (AAT) and β-galactosidase were co-instilled into the mouse lung. Of all the vectors tested rh.64R1, AAV5 and AAV6 were the most efficient. The high transduction observed in mouse was reproduced in HAE cell cultures for both rh.64R1 and AAV6 but not for AAV5. Since AAV6 was the most efficient vector in mouse and HAE we also tested the transduction efficiencies of the AAV6 singleton vectors (i.e., AAV6 variants with targeted mutations) in these models. Of these, AAV6.2 transduced mouse airway epithelium and HAE with greater efficiency than all other AAV vectors tested. We demonstrated that AAV6.2 exhibits improved transduction efficiency compared to previously reported AAVs in mouse airways and in culture models of human airway epithelium and that this vector requires further development for preclinical and clinical testing.

Received 7 August 2008; accepted 24 October 2008; published online 9 December 2008. doi:10.1038/mt.2008.261

INTRODUCTION

Delivery of therapeutic genes to the diseased airway epithelium *in vivo* is a promising option for the genetic treatment of various lung diseases that include cystic fibrosis (CF) airway disease, α-1-antitrypsin (AAT) deficiency, chronic obstructive pulmonary disease and pulmonary hypertension.[1–3] Of the many viral gene therapy vectors, adeno-associated virus (AAV)-based vectors hold great promise for efficiently targeting airway epithelium *in vivo*.[3] AAV is a single-stranded DNA virus that belongs to the *Parvoviridae* family[4] and is characterized by its safety, low toxicity, and its ability to confer long-term stable transgene expression.[5–7] Furthermore, since AAV can transduce non-dividing cells[8] its use in lung is warranted as <1% of airway epithelial cells are actively dividing.[9] AAV vectors have been developed for CF airway gene therapy[10–12] with AAVs 1, 5, and 6 transducing murine conducting airway and cultures of human ciliated airway epithelium (HAE) with varying efficiencies.[10,12–16]. However, efforts to replace a functional copy of the CF gene (*CFTR*[17]) to the lungs of CF patients using AAV2-based vectors have proven a significant challenge. Indeed, while AAV-based vectors expressing CFTR can be delivered safely to the lungs of CF patients, efficacy data have been disappointing.[18–23] To date, no correction of the bioelectric CF defect or reversal of CF pathology has been described. The development of alternative AAV serotypes is a promising strategy to improve lung-directed AAV-mediated gene transfer.

Since 2002, the AAV vector pipeline has been greatly enriched with the identification of novel AAV vector serotypes isolated from various species including caprine, avian, bovine, nonhuman primate and human.[24–26] Specifically, >110 novel AAV vector isolates have been identified in various tissues derived from human and nonhuman primate.[25,26] This effort was undertaken to further understand AAV natural ecology as well as novel biology that could improve vector technology. A safe and efficacious AAV vector is presumed to be defined by the capsid, which also impacts on gene transfer tropism, gene transfer efficiency, immune activation, and susceptibility. Thus, we have focused on defining particular AAV capsids that fulfill our criteria for candidate gene transfer vectors for the lung.

Here, we build on the existing vector discovery[25] by optimization and characterization of nineteen novel AAV vectors from isolates that are representative members of Clades A–F, as well as known serotypes AAVs 1, 2, 5, 6, 7, 8, and 9 on relevant *in vivo* and *in vitro* model systems of the human airway epithelium.

RESULTS

AAV-mediated transduction of mouse lung airway epithelium *in vivo*

AAV vectors expressing human AAT (hAAT) or nLacZ using the chicken-β-actin promoter were screened initially in the mouse lung in a single-blinded manner. Using a vector co-instillation strategy we determined the ability of the candidate AAV vectors to transduce the mouse lung by measuring hAAT secretion over time in the serum and evaluating tropism for conducting airway versus alveolar epithelium by nLacZ expression. A mixture of AAV vectors expressing hAAT and nLacZ were administered to

Correspondence: *James M. Wilson, Suite 2000, Translational Research Laboratories, 125 South 31st Street, Philadelphia, Pennsylvania 19104-3403, USA. E-mail: wilsonjm@mail.med.upenn.edu*

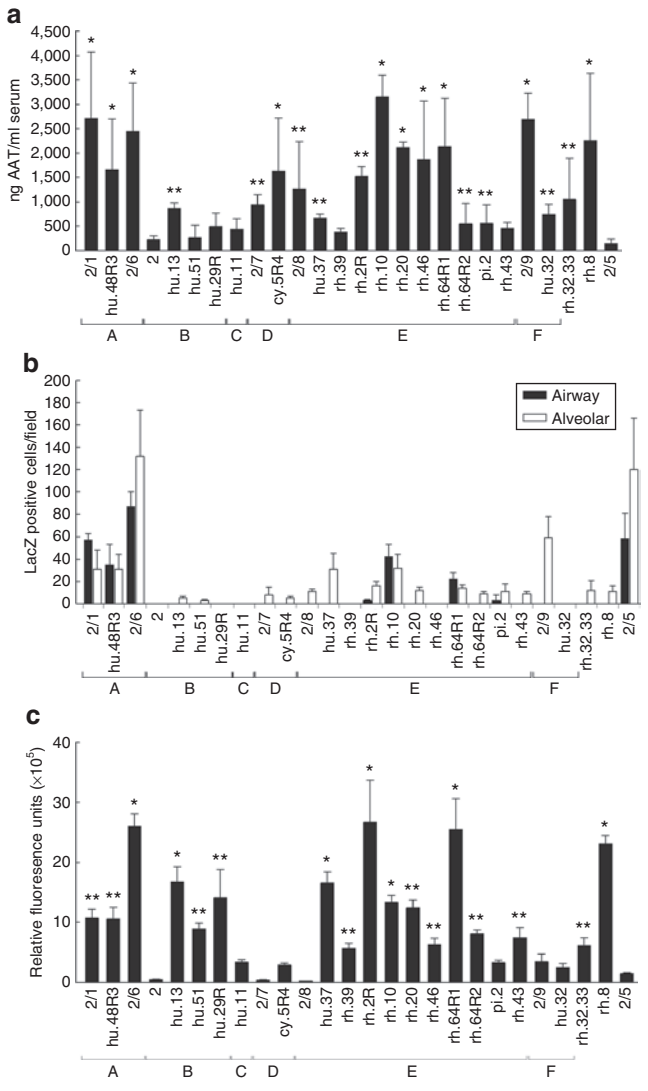

Figure 1 Transduction efficiency of the AAV vectors in model systems of airway epithelium. (**a**) hAAT expression (ng/ml) in serum 28 days following a single dose of 10^{11} genome copies of AAV vectors expressing hAAT. Results are presented as the average of $n = 15 \pm$ SD. (**b**) *nLacZ* gene transfer was quantitated by counting the number of nLacZ positive cells in mice treated with all vectors. Values presented as the average of $n = 15 \pm$ SD. Filled columns represent cells of the conducting airways and open columns represent cells of the alveolar epithelium. (**c**) *GFP* gene transfer was quantitated in representative images using the Image J software. Results are presented as the average of $n = 8$ (two cultures per vector, repeated four times) ± SD. Vectors are grouped in Clades A–F with the exception of the outliers rh.32.33, rh.8, and AAV5. *High transducers, **medium transducers, $P < 0.05$, analysis of variance, Student–Newman–Keuls test.

mice ($n = 15$) by intratracheal instillation. Twenty-eight days later, mice were killed and sera analyzed for hAAT concentration and lung tissue evaluated for nLacZ expression. Based on hAAT serum concentrations, the 19 candidate vectors, including the well established serotypes AAV1, 2, 5, 6, 7, 8, and 9, were segregated into three groups of expression levels (high, medium, and low). The high-expressors group conferring hAAT concentrations in serum >1,500 ng hAAT/ml serum were serotypes AAV1, 6, 9, and novel vectors rh.8, rh.10, rh.20, rh.46, rh.64R1, hu.48R3, and cy.5R4 (**Figure 1a**). In the medium-expressors group with 500–1,500 ng hAAT/ml serum were serotypes AAV7, 8 and novel vectors rh.2R, rh.32.33, rh.64R2, hu.13, hu.32, hu.37, and pi.2. Of note is the observation that both high and medium expressors all generated significantly higher hAAT levels than produced by AAV2 or AAV5 ($P < 0.05$, analysis of variance, Student–Newman–Keuls test, $n = 15$). In the low-expressors group (<500 ng/ml hAAT) including AAV2 and AAV5, the novel vectors rh.39, rh.43, hu.11, hu.29R, and hu.51 produced very low levels of hAAT which approximated the detection sensitivity of the hAAT ELISA.

Histochemical analyses of mouse lungs for β-galactosidase activity revealed that the candidate AAV vectors demonstrated distinct targeting patterns of transduction for either conducting airway and/or alveolar epithelium with varying degrees of efficiency (**Figure 1b**). AAVs 1, 5, and 6 have been previously shown to target mouse conducting airway in addition to alveolar epithelium[10,11,13,14] while AAV9 vectors preferentially transduce alveolar epithelium.[14] Quantitation of the number of cells of the conducting airways and alveolar cells expressing nLacZ provided a rank order of AAV vectors with AAV6 and AAV5 being the most efficient and closely followed by AAV1, rh.64R1, hu.48R3, and rh.10 (**Figure 1b**). The transduction profile in lung varied significantly between different AAV vectors. For AAV2 and hu.29R no transduced cells were detected in the conducting airway and very few or no transduced cells detected in the alveolar epithelium. LacZ transduction profiles correlated well with hAAT expression levels from the same animals, as highlighted by the consistently poor performance of AAV2 and hu.29R while the AAV6, AAV1, rh.10, hu.48R3, and rh.64R1 vectors (presented in descending order of nLacZ transduction efficiency) efficiently transferred genes to conducting airways. In addition, we found tropism differences for conducting airway and alveolar epithelium with the AAV6 vector targeting both these cell populations with almost equal efficiency whereas, AAV1, rh.10, hu.48R3, and rh.64R1 exhibited a preference for conducting airway (**Figures 1b** and **2a**). AAV9 and rh.8 showed high systemic concentrations of hAAT and LacZ transduction was limited to alveolar epithelium. For vectors that expressed either low levels of hAAT (hu.51, hu.29R, hu.11, rh.39, and rh.43), or moderate hAAT levels (AAV7, AAV8, hu.13, hu.37, rh.64R2, pi.2, hu.32, rh.2R, and rh.32.33), nLacZ transduction was observed in the alveolar epithelium but at very low amounts. We found no correlation of *hAAT* and *nLacZ* gene transfer for rh.20 and cy.5R4, which both expressed high hAAT levels, but resulted in low nLacZ transduction efficiency of alveolar epithelium only.

AAV-mediated nLacZ transduction of mouse nasal airway epithelium *in vivo*

The mouse nasal airway epithelium most closely resembles the epithelium of the human conducting airways in terms of cell composition and ion transport properties.[27,28] We evaluated the performance of all the candidate AAV vectors in the mouse nasal airway epithelium. Mice ($n = 5$/group) were inoculated intranasaly with 10^{11} genome copies of each AAV vector expressing nLacZ. *En face* examination of gross sections of the nasal passages was performed 28 days later. At this time point, the transduction profile for many of the AAV vectors was not similar to that previously shown for mouse lung. In fact, only AAVs 5, 6, 9, rh.64R1, rh.64R2 resulted in nLacZ expression in the nasal airway

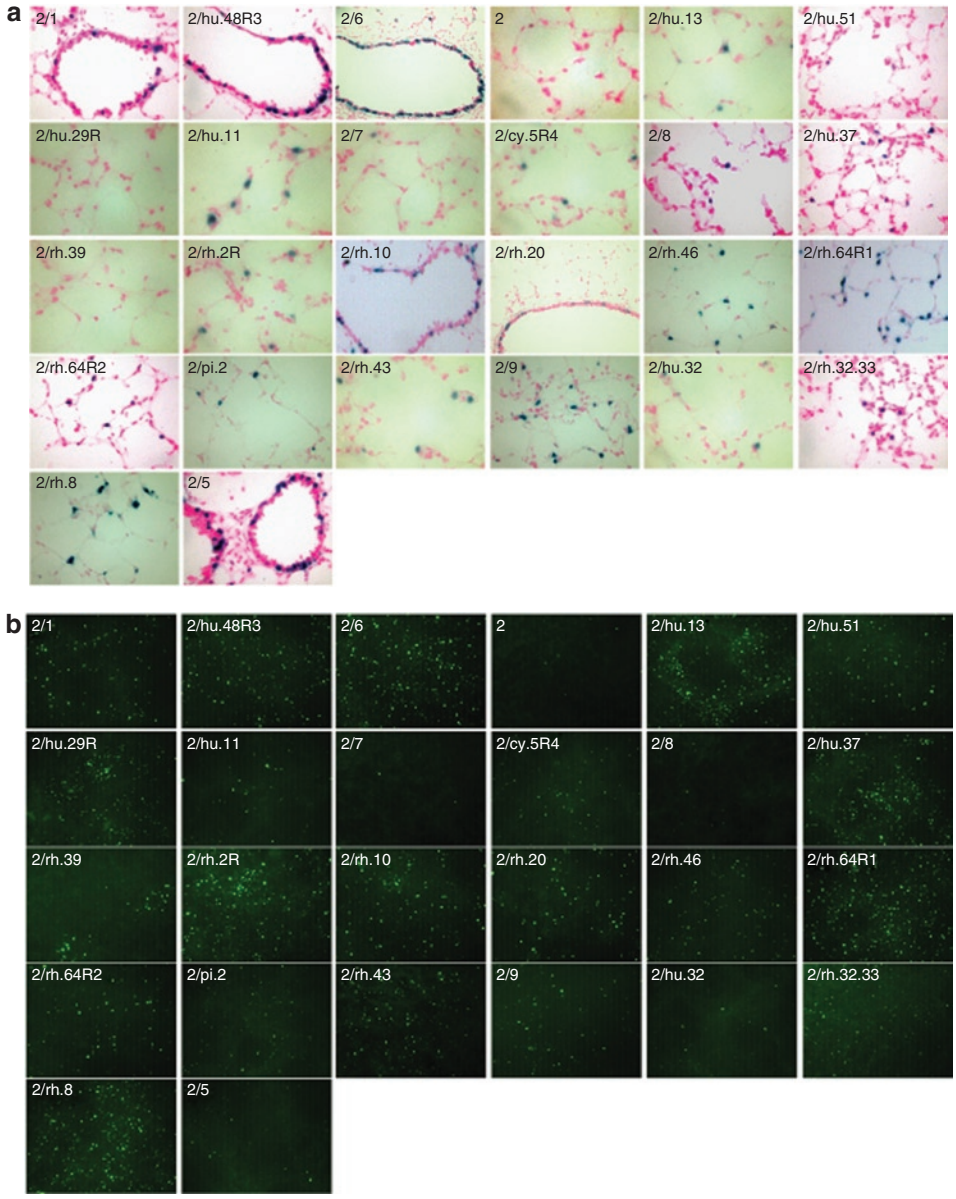

Figure 2 AAV-mediated-transgene expression in models of airway epithelium. (**a**) *nLacZ* gene transfer to mouse lung was assessed by histochemical analysis. Sections were counterstained with nuclear fast red and representative images taken at ×200 were chosen based on regions predominately showing LacZ transduction. Representative images of $n = 15$ mice are shown. (**b**) Transduction efficiency of AAV vectors in cultures of HAE. *En face* view of GFP-mediated gene transfer to HAE 7 days after apical inoculation of 10^{11} genome copies. Representative images of $n = 8$ cultures taken at ×5 are shown.

epithelium (Figure 3). Since the AAV vectors that transduced the nasal airway epithelium did not target olfactory epithelium we quantitated gene transfer only in the respiratory and transitional epithelium by counting nLacZ expressing cells in three standard cross sections (Figure 3). AAV6 and AAV5 transduced the nasal airway epithelium most efficiently and were statistically more efficient than rh.64R1, rh.64R2, and AAV9 ($P < 0.05$, analysis of variance, Student–Newman–Keuls test).

AAV-mediated GFP transduction of human airway epithelium cultures *in vitro*

The discrepancies in the transduction efficiency of the candidate AAV vectors in the epithelium of the mouse nose and mouse lung highlights the limitation of using these models to evaluate AAV vectors for human lung use and prompted the assessment of the candidate AAV vectors in more relevant models of human airway epithelium. *In vitro* models of HAE are accepted to recapitulate key aspects of the human conducting airway epithelium in terms of cell composition, tight junction formation and the formation of an overlaying airway surface liquid layer.[29] In a double-blinded fashion, all our candidate AAV vectors (expressing GFP) were applied to the apical surface of HAE (two cultures per vector) using cells derived from a total of four patients. Using several different production lots of the AAV vectors representative transduction efficiency data seven days after vector inoculation are presented in Figure 2b. Based on GFP expression, vectors were scored as

high-, medium-, or low-transducers. XZ confocal analysis demonstrated the presence of GFP ciliated and nonciliated cells in cultures infected with either the high- or medium-transducers (data not shown). The high-transducing vector group included AAV6, hu.13, hu.37, rh.2R, rh.10, rh.64R1, and rh.8 (**Figures 1c** and **2b**). The medium-transducing group was comprised of AAV1, hu.48R3, hu.51, hu.29R, rh.39, rh.20, rh.46, rh.64R2, rh.43, and rh.32.33 (**Figures 1c** and **2b**). The low-transducing group included hu.11, cy.5R4, pi.2, and AAV9 (**Figures 1c** and **2b**). Very few positive GFP cells were seen in HAE inoculated with AAVs 2, 7, 8, hu.32 and AAV5 (**Figures 1c** and **2b**).

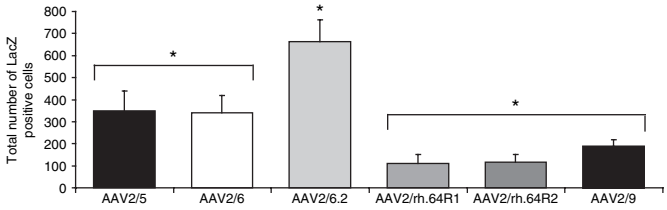

Figure 3 AAV-mediated-*nLacZ* gene transfer to mouse nose. The number of nLacZ positive cells was quantitated and is presented as the average of $n = 5 \pm$ SD. $^*P < 0.05$, analysis of variance, Student–Newman–Keuls test.

Evaluation of the AAV6-based singleton AAV vectors

The consistent superior performance of AAV6 in the epithelial cells of mouse lung (**Figures 1b** and **2a**), mouse nose (**Figure 3**) and HAE (**Figure 2b**) prompted the evaluation of the AAV6 singleton mutants in these model systems.[30,31] Briefly, the AAV6.1 vector was created by mutating the lysine (K) residue at position 531 to glutamic acid (E) ablating the heparin binding motif. The AAV6.2 vector mutant was created by mutating the phenyalanine (F) residue at position 129 to leucine (L), maintaining the heparin binding motif. The K and the F residues were also mutated together to create the AAV6R2 vector.

The efficiencies of AAV6 and the singleton mutants were tested in mouse lungs following an intratracheal co-instillation of each vector expressing nLacZ and hAAT using protocols identical

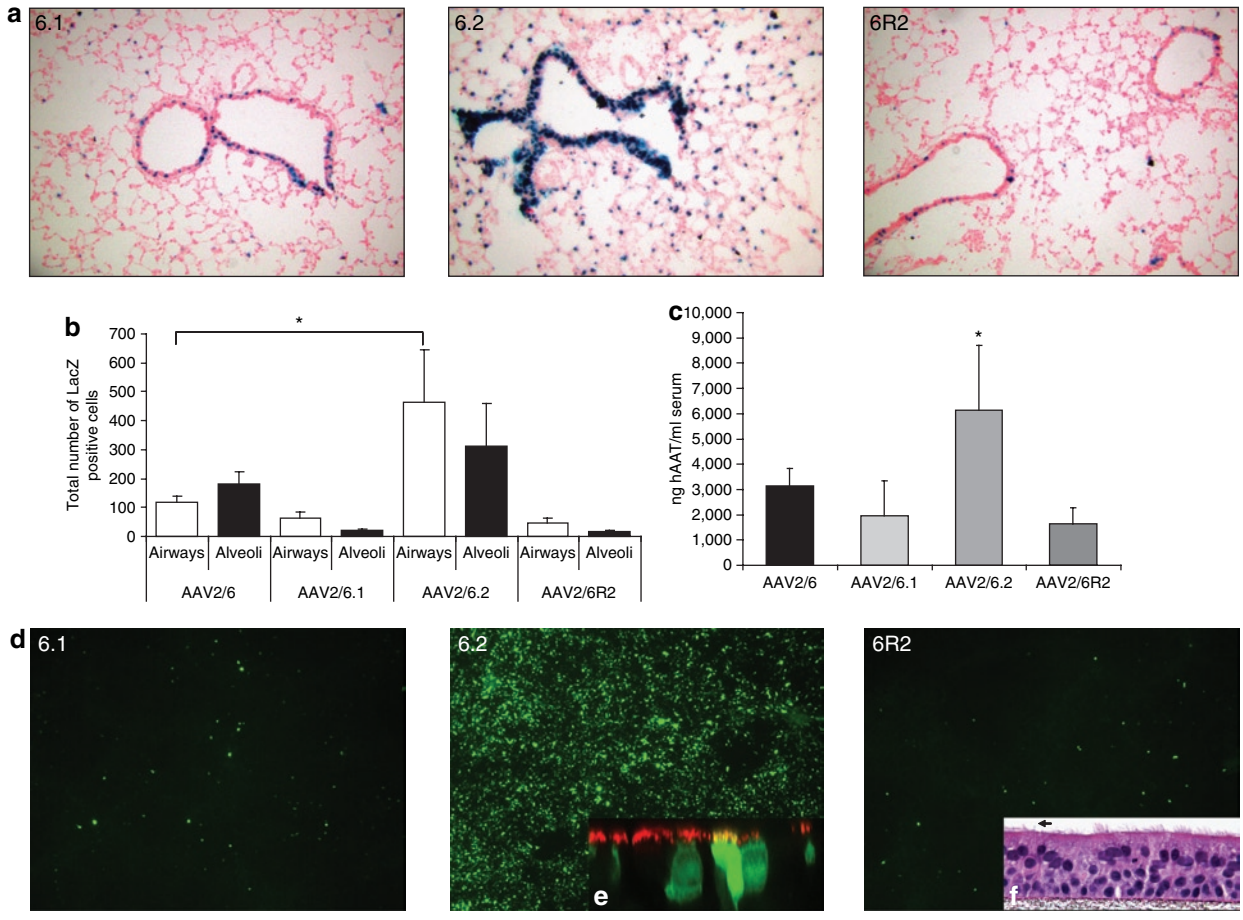

Figure 4 Transduction efficiency of AAV6 vector singleton mutants. (**a**) *nLacZ* gene transfer to mouse lung was assessed by histochemical analysis of the AAV6.1, AAV6.2, and AAV6R2 vector-treated mouse lungs. Representative images of $n = 15$ mice taken at ×100 are shown. (**b**) The number of nLacZ positive cells was quantitated and is presented as the average of $n = 15 \pm$ SD. Open columns represent epithelial cells of the conducting airways and filled columns represent epithelial cells of the alveolus. (**c**) hAAT expression (ng/ml) in serum 28 days following a single dose of 10^{11} genome copies of the AAV6 singleton mutant vectors expressing hAAT. Results are presented as the average of $n = 15 \pm$ SD. (**d**) *En face* view of GFP-gene transfer to HAE mediated by AAV6.1, AAV6.2, and AAV6R2 7 days after inoculation. (**e**) XZ confocal image of AAV6.2 inoculated HAE with cilia immunostained red (see Materials and Methods for detail). Both ciliated and nonciliated cells were transduced by AAV vectors. (**f**) Hematoxylin–eosin staining of representative cross section of human airway epithelial cultures showing presence of ciliated cells (black arrow). $^*P < 0.05$, analysis of variance, Student–Newman–Keuls test.

to those described earlier. Compared to the parent AAV6 vector the AAV6.1 vector maintained tropism for conducting airway and alveolar epithelium (**Figure 4a**) but its transduction efficiency was severely hampered. Interestingly, the transduction efficiency of AAV6.1 resembled that of AAV1 (**Figures 1** and **2**). The transduction efficiency of AAV6.2 in mouse conducting airway (**Figure 4a**) and nasal airway (**Figure 3**) was superior to any other vector and was statistically better than AAV6, AAV6.1, and AAV6R2 ($P < 0.05$, analysis of variance, Student–Newman–Keuls test). AAV6.2 also produced the highest amount of hAAT in serum compared to all the other AAV vectors, including AAV6.1 and AAV6R2 (**Figures 1a** and **4c**). Although AAV6.2 was significantly better than AAV6 in generating hAAT, the difference in transduction efficiency was not as dramatic as that observed by nLacZ transduction.

The AAV6 vector singletons were tested on HAE and we found that both AAV6.1 and AAV6R2 resulted in low transduction efficiency (**Figure 4d**) similar to that produced by AAV5, hu.11 and hu.32 (**Figures 1c** and **2b**). However, AAV6.2 consistently resulted in almost 20% GFP transduction ($3.12 \pm 0.86 \times 10^7$ relative fluorescence units, $n = 8$) of luminal epithelial cells in cultures derived from six different patients. Both ciliated and nonciliated epithelial cells were detected as GFP-positive by XZ confocal microscopy, (**Figure 4e**). AAV6.2 was consistently more efficient than AAV6 at transducing HAE (**Figures 1c** and **2b**).

DISCUSSION

To identify candidate AAV vectors for pre-clinical and clinical assessment for diseases of the lung we have characterized novel AAV vectors isolated from different tissues including human and nonhuman primates. Nineteen novel or engineered AAV vectors representing members of Clades A–F and several Clade outliers (rh.8, rh.32.33, and AAV5) were evaluated in relevant airway epithelium model systems. These studies revealed that AAV capsid structure has a significant impact on airway transduction efficiency and tropism for conducting airway and/or alveolar epithelium.

Overall, Clade A–based vectors were the most efficient at transducing the mouse conducting airway with AAV6 and the singleton mutant AAV6.2 (**F**129**L**) performing the best. The ability of AAV6 to bind to heparin has been implicated in improved binding, but not uptake, by airway epithelial cells.[10] Surprisingly, competition experiments have demonstrated that heparin binding on AAV6 is not required for infection.[10] Indeed, in our studies we found that for AAV6.2, which maintained heparin binding, there was increased transduction in all model systems studied, suggesting that binding was significantly improved. Preservation of the AAV6 heparin binding domain has also been shown to be important in sustaining efficient transduction of mouse liver.[31] Ablation of the heparin binding motif in the AAV6 singleton mutants, AAV6.1 and AAV6R2, was detrimental to efficient gene transfer to mouse airways and HAE. Indeed, capsids from other Clade A viruses, that lack the lysine residue at position 531 implicated in heparin binding,[31] including AAV1, hu.48R3, AAV6.1, and AAV6R2, were significantly less efficient at transducing the conducting airways and HAE compared to AAV6 and AAV6.2. Although Clade A vectors efficiently produced high levels of hAAT protein following intratracheal instillation to lung, a clear correlation with respect to heparin binding and transduction of alveolar and nasal airway epithelium was highlighted. The heparin binders AAV6 and AAV6.2 transduced the alveolar and nasal airway epithelium efficiently while the nonheparin binders AAV1, hu.48R3, AAV6.1, and AAV6R2 were inefficient in these tissues.

In contrast, Clade B vectors, which include the prototype AAV2, contrary to previous reports[12] failed to transduce mouse conducting and HAE and also did not transduce the mouse nasal airway. Similarly, hu.51 (ref. 32), an AAV2-like vector that binds heparin, was also inefficient in all model systems tested. However, hu.13 and hu.29R (which do not bind heparin) did transduce HAE with good efficiency. Hu.13 and hu.51 (which bind heparin) also transduced, albeit at low levels, mouse alveolar epithelium potentially explaining the low to moderate levels of serum hAAT protein levels measured with these vectors. In this instance, absence of heparin binding significantly improved the vector transduction profile in HAE. The stark contrast of the impact of heparin affinity on transduction efficiency between Clade A and Clade B vectors demonstrates the context dependent requirements for AAV permissivity.

We only evaluated one vector representing Clade C, hu.11. This vector poorly transduced mouse alveolar epithelium and HAE. Members of Clade F transduced mouse alveolar epithelium with moderate (hu.32) to good (AAV9) efficiency but were poorly efficient in HAE. The difference in performance between hu.32 and AAV9 is interesting since they only differ structurally within the VP12-unique domains and have identical VP3 domains on their capsid. The most inconsistent transduction patterns in lung were seen with vectors of Clade E, which includes AAVs 7 and 8. Most of the vectors evaluated resulted in moderate to good serum hAAT protein expression and with the exception of rh.39, all vectors transduced mouse alveolar epithelium with rh.64R1 being the most efficient. Rh.10 and rh.64R1 were as efficient as AAV1 at transducing the mouse conducting airway and HAE. Rh.64R1 and rh.64R2 were the only Clade E members that transduced the mouse nasal airway epithelium. Interestingly, while rh.64R2 transduced the nasal airway epithelium it failed to transduce conducting airway suggesting that even slight changes to the capsid structure can affect cell tropism.

AAV5 has been shown to effectively target the mouse conducting and nasal airway epithelium;[12–14] however in contrast to the findings of Zabner and colleagues,[12,15] we found that AAV5 was poorly efficient when applied apically to HAE. AAV5 was also shown to be significantly more effective at transducing mouse conducting airway than AAV1,[13] which is in agreement with our findings in mouse airway and HAE. In a different study comparing AAV1 and AAV5 in mouse lung, Virella-Lowell and colleagues demonstrated that AAV1 did not efficiently transduce mouse conducting airways.[16] However, the authors did show that AAV1 and AAV5 were equally efficient in mouse lung using luminescence as a readout.[16] The disadvantage of luminescence imaging is that it does not distinguish vector tropism such as differential transduction of conducting airways and alveolar epithelium. Seiler and colleagues have shown that AAV6 was more effective than AAV5 at transducing mouse conducting epithelium *in vivo*.[15]

Table 1 Decision matrix for selection of lead AAV vector candidates

		Mouse lung			Mouse nose	Human airway cultures	
		LacZ gene expression		*hAAT* gene expression	*LacZ* gene expression	*GFP* gene expression	
Clade	Capsid	Conducting Airways WP 2	Alveoli WP 1	WP 2	WP 3	WP 4	Score
A	AAV1	2	1	3	—	2	19
	hu.48R3	2	1	3	—	2	19
	AAV6	4	3	4	3	2	36
	AAV6.2	5	5	5	4	4	53
	AAV6.1	2	1	3	—	0.5	13
	AAV6R2	2	1	3	—	0.5	13
B	AAV2	—	—	1	—	—	2
	hu.13	—	1	2	—	2	13
	hu.51	—	1	1	—	1	7
	hu.29R	—	—	1	—	2	10
C	hu.11	—	1	1	—	0.5	5
D	AAV7	—	1	2	—	0.5	7
	cy.5R4	—	1	3	—	0.5	9
E	AAV8	—	1	2	—	0.5	7
	hu.37	—	1	2	—	2	13
	rh.39	—	—	1	—	0.5	4
	rh.2R	—	1	2	—	2	13
	rh.10	2	1	3	—	2	19
	rh.20	2	1	3	—	2	19
	rh.46	—	1	3	—	0.5	9
	rh.64R1	2	1	2	2	2	23
	rh.64R2	—	1	2	2	0.5	13
	pi.2	1	1	2	—	0.5	9
	rh.43	—	1	1	—	2	11
F	AAV9	—	2	3	2	0.5	16
	hu.32	—	1	2	—	1	9
	rh.32.33	—	1	2	—	0.5	7
	rh.8	—	1	3	—	2	15
	AAV5	3	3	1	3	0.5	22

The model systems on which the adeno-associated virus (AAV) vectors were assessed were assigned a weighted parameter (WP) number based on their relevance to human conducting airway. The greater their relevance to human airway, the higher the weighted parameter number. Human ciliated airway epithelium (HAE) closely models human conducting airway and was assigned the highest weighted parameter of 4 followed by mouse nasal airway that was assigned a weighted parameter of 3 and mouse conducting airway transduction that was assigned a weighted parameter of 2. AAV-mediated human α-1-antitrypsin expression following delivery to lung was assigned a weighted parameter of 2. Mouse alveolar epithelium was given the lowest weighted parameter of 1. The final score was calculated by the addition of scores based on the performance of AAV vectors on each model system after multiplication by the weighted parameter. Maximum score based on superior vector performance on all model systems is 60.

Discrepancies in transduction efficiencies between the model systems we have tested serves to highlight the difficulty in predicting the usefulness of gene transfer vectors for human use. Without testing vectors in several relevant model systems caution is required in identifying the appropriate vectors to carry into preclinical development. We have attempted to use the most accepted and most relevant model systems of the airway epithelium for our studies including the mouse upper and lower airways *in vivo* and HAE *in vitro*. The use of these models has led to the identification of AAV6.2 as the most efficacious AAV vector that can now be carried into further preclinical and possibly clinical testing.

A challenge in the development of lung-directed gene transfer for humans has been the uncertainty of the relevance of the different model systems of airway. Most studies utilize the mouse lung to assess gene transfer agents although the mouse nasal epithelium is proposed to most represent the morphology and physiology of the human conducting airway epithelium.[14,33] While rabbits and macaques have been used for lung-directed gene transfer studies, species susceptibility to virus-based vector-mediated transduction has limited their wide application.[3,34–36] However, it is clearly important to exercise caution when translating findings from animal models to predictions for promising vectors in clinical trials.

The *in vitro* model of HAE bring us one step closer to studying gene transfer vectors in human airways *in vivo* since these models recapitulate key aspects of the human conducting airway epithelium cell composition and physiology. We discovered that AAV vectors (i.e., rh.2R, hu.13, hu.29R) that did not transduce mouse nasal epithelium or were poorly efficient in mouse lung were as effective as AAV6 at transducing HAE. These findings clearly exemplify the need to evaluate gene transfer agents in different but relevant model systems of the airway to grade AAV vectors for selection of lead clinical vector candidates.

The current *in vivo* and *in vitro* model systems of the airway represent certain aspects of the human conducting airway. In an effort to identify an AAV vector for preclinical and possibly clinical testing it became apparent that much like the field of small molecule drug screening, it is crucial to assess the performance of vectors in multiple model systems. Here, we propose a decision matrix based on criteria that identify lead vectors for lung directed gene transfer. While there are obvious limitations to all of the model systems we used (mouse nose, mouse lung or cultures of HAE) taken together they can provide invaluable insight into vector biology and tropism. Weighted parameters were used in a decision making algorithm to reflect the importance or relevance of certain model systems more appropriately. In the context of our studies and the models of airway epithelium that we evaluated, HAE closely models human conducting airway and as such, was assigned the highest weighted parameter of 4. Mouse nasal airway was assigned a weighted parameter of 3 and mouse conducting airway a weighted parameter of 2. Interestingly, using hAAT in mouse lung was frequently indicative of the transduction efficiency of vectors in HAE and as such, was assigned a weighted parameter of 2. nLacZ transduction of mouse alveolar epithelium was given the lowest weighted parameter of 1 (**Table 1**). The gene transfer efficiency of the AAV vectors in each model system was scored on a scale of 1–5 (5 being the most efficient). Using this decision matrix, and the weighted parameter values assigned to each model system we found AAV6.2 to be the most promising AAV vector for human conducting airway transduction. In brief, as shown in **Table 1**, AAV6.2 scored 5 for nLacZ transduction of mouse conducting airway (score 5 × 2 = 10), 5 for nLacZ transduction of mouse alveolar epithelium (score 5 × 1 = 5), 5 for hAAT expression (score 5 × 2 = 10), 4 for nLacZ transduction of mouse nasal airway (score 4 × 3 = 12) and 4 for GFP transduction of HAE (score 4 × 4 = 16), and was assigned a total score of 53 [(10) + (5) + (10) + (12) + (16)] from a maximum of 60. We propose that AAV6.2 has fulfilled the requirements that warrant this vector being taken further into testing for potential AAV gene therapeutics for CF lung disease, AAT deficiency or chronic obstructive pulmonary disease.

MATERIALS AND METHODS

DNA constructs and vector production. The AAV vectors flanked with AAV2 inverted terminal repeats (ITRs) contained either (i) a *LacZ* gene fused to a nucleus localization sequence at the N-terminus (*nLacZ*); (ii) an enhanced green fluorescence protein gene; or (iii) an *hAAT* gene under the transcriptional control of the cytomegalovirus–enhanced chicken-β-actin or the cytomegalovirus promoter. All AAV sequences of natural isolates were deposited in Genbank. Most *wild type* isolate sequences were previously described.[24,25,37] In brief, natural AAV isolates from human and nonhuman primate sources were optimized for vector production and gene transfer efficiency by modifying certain atypical capsid residues called singletons to their conserved state.[32] Those particular residues were identified by structure-function analyses. Modifications were achieved by site-directed mutagenesis (Quickchange, Stratagene, LaJolla, CA) for all but one of the capsid sequences and confirmed by sequencing. Rh.32.33 was generated by cloning a BsiWI–BbvCI restricted rh.33 fragment into AAV2/rh.32. Novel capsid sequences were transferred from a PCR isolate into TOPO cloning vectors (Invitrogen, Carlsbad, CA) and then subsequently cloned into an AAV packaging construct by partial restriction digestion at the first internal *cap* XhoI site on the 5′ end and, depending on the orientation in the TOPO plasmid, a PmeI or EcoRV blunt end digestion. AAV vectors were produced as previously described.[37,38]

Animal models. C57BL/6 mice (6–8 weeks) were purchased from Charles River Laboratories (Wilmington, MA) and kept at the Animal Facility of the Translational Research Laboratories at the University of Pennsylvania. Mice were anesthetized using an intraperitoneal injection of ketamine/xylazine. Using previously described methods mice were inoculated by intranasal or intratracheal routes with 10^{11} genome copies of AAV vector in a final volume of 50 µl PBS as previously described.[14] Control-treated animals received PBS only. All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Gene expression. For *nLacZ* gene expression, the lungs were processed as previously described.[14] Transduction efficiency was estimated by examining 20 high–power (×100) fields from two cryosections spaced 200 µm apart and data presented as nLacZ expressing cells per ×100 field. Assessment of gene transfer to the nasal airway epithelium was performed as described previously and is presented as the total number of LacZ positive cells in the respiratory epithelium as quantitated in three standard cross-sections of nasal airway.[28] For hAAT detection, serum samples were collected by retro-orbital bleeding and concentration determined by ELISA.[25] For GFP expression, each image was analyzed using ImageJ software (Rasband 1997–2006; National Institutes of Health, Bethesda, MD, http://rsb.info.nih.gov/ij/). For each brightness value between 60 (which is above background) and 255 (the maximum value), the number of corresponding pixels was determined. The number of pixels was then multiplied with their brightness value and the products added to give a final value for GFP expression.

Air–liquid interface cultures of HAE. Fresh donor human tracheobronchial tissues were obtained from NDRI and airway epithelial cells isolated by the University of North Carolina at Chapel Hill Cystic Fibrosis Center Cell Culture Core according to published procedures.[29,39] Freshly isolated airway epithelial cells were seeded at 250,000 per cm^2 on permeable membrane supports (Millicells PICM 01250, 0.4 µm pore size, Millipore, Billerica, MA), and allowed to reach confluency, upon which the cultures were maintained at an air–liquid interface to allow differentiation into pseudostratified ciliated airway epithelium. The HAE cultures were maintained at an air–liquid interface for 4–6 weeks before inoculation with AAV vectors.

Inoculation of cultures of HAE. For viral inoculation, the apical surfaces of HAE cultures were briefly rinsed with PBS to remove accumulated apical secretions, followed by inoculation with 10^{11} genome copies (MOI = 200,000) of candidate AAVGFP vectors in 100 µl. All AAV vector candidates expressed GFP using the cytomegalovirus promoter. Virus inoculum was removed 2 hours later and cultures maintained at 37 °C. The extent of AAV-mediated GFP transduction was monitored for each of the 7 days after inoculation by observing GFP-positive cells using an inverted fluorescent microscope, Leica DMIRB (Leica Microsystems, Bannockburn, IL, USA). Fluorescent photomicrographs were obtained using a color CCD camera (Q-Imaging RET-EXI-F-M-12C, Q-Imaging, Surrey, BC, Canada), and the Q-capture software. To identify transduced epithelial cell types,

HAE cultures were fixed in 4% paraformaldehyde and cilia immunolabelled with a β-tubulin IV specific monoclonal antibody (Sigma, St. Louis, MO) and fluorescent XZ confocal images obtained with a Zeiss 510 Meta Laser Scanning Confocal Microscope as described previously.[40]

Statistical analysis. Statistical analysis was performed using the SigmaStat 3.1 program (SPSS, Chicago, IL). Statistical significance was set at $P < 0.05$ and statistical power at 0.80. Results are presented as the average of 2 sets of pooled samples ± SD. Student's *t*-test was used for two-group comparisons and analysis of variance Student–Newman–Keuls test for multiple group comparisons.

ACKNOWLEDGMENTS

We thank Deirdre McMenamin and Regina Munden for invaluable assistance with animal studies (Gene Therapy Program); Peter Bell and Di Wu (Gene Therapy Program) for tissue sectioning; Julie Johnston and Arbans Sandhu (Penn Vector) for supplying vectors; the Directors and Teams of the UNC Cystic Fibrosis Center Tissue Culture Core, the Morphology and Morphometry Core for supplying reagents and technical expertise and Ms. Susan Burkett for technical assistance. We thank Jiyang Shen for developing a macro program to allow analysis of GFP expression of the transduced HAEC cells. This work was supported by the following grants CFF R881 (J.M.W.), P01-HL051746 (J.M.W.), GSK (J.M.W.), R01 HL77844 (R.J.P.), P01 HL051818 (R.J.P.), P30 DK065988 (R.J.P.). J.M.W. is an inventor on patents licensed to various biopharmaceutical companies.

REFERENCES

1. Aneja, MK and Rudolph, C (2006). Gene therapy of surfactant protein B deficiency. *Curr Opin Mol Ther* **8**: 432–438.
2. Cruz, PE, Mueller, C and Flotte, TR (2007). The promise of gene therapy for the treatment of α-1 antitrypsin deficiency. *Pharmacogenomics* **8**: 1191–1198.
3. Flotte, TR, Ng, P, Dylla, DE, McCray, PB Jr, Wang Kolls, JK and Hu, J *et al.* (2007). Viral vector-mediated and cell-based therapies for treatment of cystic fibrosis. *Mol Ther* **15**: 229–241.
4. Muzyczka, N and Burns, KI (2001). *Parvoviridae: The Viruses and Their Replication.* Lippincott Williams & Wilkins: Philadelphia.
5. Carter, BJ (2004). Adeno-associated virus and the development of adeno-associated virus vectors: a historical perspective. *Mol Ther* **10**: 981–989.
6. Duan, D, Sharma, P, yang, J, Yue, Y, Dudus, L, Zhang, Y *et al.* (1998). Circular intermediates of recombinant adeno-associated virus have defined structural characteristics responsible for long-term episomal persistence in muscle tissue. *J Virol* **72**: 8568–8577.
7. Flotte, TR and Laube, BL (2001). Gene therapy in cystic fibrosis. *Chest* **120**: 124S–131S.
8. Russell, DW, Miller, AD and Alexander, IE (1994). Adeno-associated virus vectors preferentially transduce cells in S phase. *Proc Natl Acad Sci USA* **91**: 8915–8919.
9. Leigh, MW, Kylander, JE, Yankaskas, JR and Boucher, RC (1995). Cell proliferation in bronchial epithelium and submucosal glands of cystic fibrosis patients. *Am J Respir Cell Mol Biol* **12**: 605–612.
10. Halbert, CL, Allen, JM and Miller, AD (2001). Adeno-associated virus type 6 (AAV6) vectors mediate efficient transduction of airway epithelial cells in mouse lungs compared to that of AAV2 vectors. *J Virol* **75**: 6615–6624.
11. Halbert, CL, Lam, SL and Miller, AD (2007). High-efficiency promoter-dependent transduction by adeno-associated virus type 6 vectors in mouse lung. *Hum Gene Ther* **18**: 344–354.
12. Zabner, J, Seiler, P, Yang, J, Walters, R, Kotin, RM, Filgeras, W *et al.* (2000). Adeno-associated virus type 5 (AAV5) but not AAV2 binds to the apical surfaces of airway epithelia and facilitates gene transfer. *J Virol* **74**: 3852–3858.
13. Auricchio, A, O'Conner, E, Weiner, D, Gao, GP, Hildinger, M, Wang, L *et al.* (2002). Noninvasive gene transfer to the lung for systemic delivery of therapeutic proteins. *J Clin Invest* **110**: 499–504.
14. Limberis, MP and Wilson, JM (2006). Adeno-associated virus serotype 9 vectors transduce murine alveolar and nasal epithelia and can be readministered. *Proc Natl Acad Sci USA* **103**: 12993–12998.
15. Seiler, MP, Miller, AD, Zabner, J and Halbert, CL (2006). Adeno-associated virus types 5 and 6 use distinct receptors for cell entry. *Hum Gene Ther* **17**: 10–19.
16. Virella-Lowell, I, Zusman, B, Foust, K, Loiler, S, Conlon, T, Song, S *et al.* (2005). Enhancing rAAV vector expression in the lung. *J Gene Med* **7**: 842–850.
17. Riordan, JR, Rommen, JM, Kermem, B, Alon, N, Rozmahel, R, Grzelczak, Z *et al.* (1989). Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science* **245**: 1066–1073.
18. Aitken, ML, Moss, RB, Waltz, DA, Dovey, ME, Tonelli, MR, McNamara, SC *et al.* (2001). A phase I study of aerosolized administration of tgAAVCF to cystic fibrosis subjects with mild lung disease. *Hum Gene Ther* **12**: 1907–1916.
19. Flotte, TR, Zeitlin, PL, Reyonlds, TC, Heald, AE, Pedersen, P, Beck, S *et al.* (2003). Phase I trial of intranasal and endobronchial administration of a recombinant adeno-associated virus serotype 2 (rAAV2)-CFTR vector in adult cystic fibrosis patients: a two-part clinical study. *Hum Gene Ther* **14**: 1079–1088.
20. Moss, RB, Milla, C, Columbo, J, Accurse, F, Zeiltin, PL, Clancy, JP *et al.* (2007). Repeated aerosolized AAV-CFTR for treatment of cystic fibrosis: a randomized placebo-controlled phase 2B trial. *Hum Gene Ther* **18**: 726–732.
21. Wagner, JA, Messner, AH, Moran, ML, Daifuku, R, Kouyama, K, Desch, JK *et al.* (1999). Safety and biological efficacy of an adeno-associated virus vector-cystic fibrosis transmembrane regulator (AAV-CFTR) in the cystic fibrosis maxillary sinus. *Laryngoscope* **109**: 266–274.
22. Wagner, JA, Nepomuceno, IB, Messner, AH, Moran, ML, Balson, EP, Dimiceli, S *et al.* (2002). A phase II, double-blind, randomized, placebo-controlled clinical trial of tgAAVCF using maxillary sinus delivery in patients with cystic fibrosis with antrostomies. *Hum Gene Ther* **13**: 1349–1359.
23. Wagner, JA, Reynolds, JA, Moran, ML, Moss, RM, Wine, JJ, Flotte, TR *et al.* (1998). Efficient and persistent gene transfer of AAV-CFTR in maxillary sinus. *Lancet* **351**: 1702–1703.
24. Gao, G, Alvira, MR, Somanathan, S, Lu, Y, Vandenberghe, LH, Rux, JJ *et al.* (2003). Adeno-associated viruses undergo substantial evolution in primates during natural infections. *Proc Natl Acad Sci USA* **100**: 6081–6086.
25. Gao, G, Vandenberghe, LH, Alvira, MR, Lu, Y, Calcedo, R, Zhou, X *et al.* (2004). Clades of Adeno-associated viruses are widely disseminated in human tissues. *J Virol* **78**: 6381–6388.
26. Gao, G, Vandenberghe, LH and Wilson, JM (2005). New recombinant serotypes of AAV vectors. *Curr Gene Ther* **5**: 285–297.
27. Grubb, BR and Boucher, RC (1999). Pathophysiology of gene-targeted mouse models for cystic fibrosis. *Physiol Rev* **79**: S193–S214.
28. Limberis, M, Anson, DS, Fuller, M and Parsons, DW (2002). Recovery of airway cystic fibrosis transmembrane conductance regulator function in mice with cystic fibrosis after single-dose lentivirus-mediated gene transfer. *Hum Gene Ther* **13**: 1961–1970.
29. Gray, TE, Guzman, K, Davis, CW, Abdullah, LH and Nettesheim, P (1996). Mucociliary differentiation of serially passaged normal human tracheobronchial epithelial cells. *Am J Respir Cell Mol Biol* **14**: 104–112.
30. Royo, NC, Vandenberghe, LH, Ma, JY, Hausourg, A, Yu, L, Maronski, M *et al.* (2008). Specific AAV serotypes stably transduce primary hippocampal and cortical cultures with high efficiency and low toxicity. *Brain Res* **1190**: 15–22.
31. Wu, Z, Asokan, A, Grieger, JC, Govindasamy, L, Agbandje-McKenna, M and Samulski, RJ (2006). Single amino acid changes can influence titer, heparin binding, and tissue tropism in different adeno-associated virus serotypes. *J Virol* **80**: 11393–11397.
32. Vandenberghe, LH, Wang, L, Somanathan, S, Xhi, Y, Figueredo, J, Calcedo, R *et al.* (2006). Heparin binding directs activation of T cells against adeno-associated virus serotype 2 capsid. *Nat Med* **12**: 967–971.
33. Parsons, DW, Hopkins, PJ, Bourne, AJ, Boucher, RC and Martin, AJ (2000). Airway gene transfer in mouse nasal-airways: importance of identification of epithelial type for assessment of gene transfer. *Gene Ther* **7**: 1810–1815.
34. Conrad, CK, Allen, SS, Afione, SA, Reyonlds, TC, Beck, SE, Fee-Maki, M *et al.* (1996). Safety of single-dose administration of an adeno-associated virus (AAV)-CFTR vector in the primate lung. *Gene Ther* **3**: 658–668.
35. Engelhardt, JF, Simon, RH, Yang, Y, Zepeda, M, Webber-Pendleton, S, Doranz, B *et al.* (1993). Adenovirus-mediated transfer of the CFTR gene to lung of nonhuman primates: biological efficacy study. *Hum Gene Ther* **4**: 759–769.
36. Fischer, AC, Beck, SE, Smith, CI, Laube, BL, Askins, FB, Guggino, SE *et al.* (2003). Successful transgene expression with serial doses of aerosolized rAAV2 vectors in rhesus macaques. *Mol Ther* **8**: 918–926.
37. Gao, GP, Alvira, MR, Wang, L, Calcedo, R, Johnston, J and Wilson, JM (2002). Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. *Proc Natl Acad Sci USA* **99**: 11854–11859.
38. Wang, L, Calcedo, R, Nichols, TC, Bellinger, DA, Dillow, A, Verma, IM *et al.* (2005). Sustained correction of disease in naive and AAV2-pretreated hemophilia B dogs: AAV2/8-mediated, liver-directed gene therapy. *Blood* **105**: 3079–3086.
39. Pickles, RJ, McCarty, D, Matsui, H, Hart, PJ, Randell, SH and Boucher, RC (1998). Limited entry of adenovirus vectors into well-differentiated airway epithelium is responsible for inefficient gene transfer. *J Virol* **72**: 6014–6023.
40. Zhang, L, Bukreyev, A, Thompson, CL, Watson, B, Peeples, ME, Collins, PL *et al.* (2005). Infection of ciliated cells by human parainfluenza virus type 3 in an *in vitro* model of human airway epithelium. *J Virol* **79**: 1113–1124.